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The role of MAPK signalling pathways in acetic acid-induced cell death of *Saccharomyces cerevisiae*

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DECLARAÇÃO

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Summary

Mitogenic Activated Protein Kinase (MAPK) cascades are important signalling pathways that allow yeast cells to swiftly adapt to changing environmental conditions. Previous studies suggested that the High Osmolarity Glycerol (HOG) MAPK pathway and ceramide production are involved in acetic-acid induced apoptosis in yeast. Evidence that changes in the levels of endogenous ceramides can affect yeast cell fate has also been put forth. However, knowledge on the molecular basis of acetic acid induced cell death and ceramide-induced cell changes, as well as the signalling pathways involved in these processes, is still lacking.

In this work, we tried to elucidate the role of MAPK signalling pathways in the cell response to ceramide and in acetic acid-induced cell death. Cells treated with the soluble ceramide analog N-acetyl-D-sphingosine (C2-ceramide) maintained plasma membrane integrity and did not produce reactive oxygen species detectable by DHE staining. A subpopulation of cells died when exposed to C2-ceramide but the surviving part eventually grew, so no reduction in plate counts was observed. Preliminary results indicated that deletion mutants in MAPK components showed smaller sensitive sub-populations than the reference strain and were therefore deemed to be more resistant to C2-ceramide, namely *wsc3*Δ. The MAPK mutants *wsc2*Δ, *wsc3*Δ, *ste20*Δ, *slt2*Δ and *mkk1*Δ/*mkk2*Δ were significantly more resistant to acetic acid than the reference strain. These mutants also displayed a lower percentage of cells with increased accumulation of ROS and a higher percentage of cells with preserved plasma membrane integrity than the wild type strain. The *WSC2* and *WSC3* genes encode sensors in the cell wall integrity MAPK pathway, suggesting a role for this signalling pathway in both ceramide and acetic acid induced cell death. The link between acetic acid-induced cell death and cell wall integrity is not known, but lipid peroxidation by acetic acid may be a plausible candidate.

Sumário

As cascatas “Mitogenic Activated Protein Kinase” (MAPK) são vias de sinalização importantes que permitem que as células de levedura se adaptem rapidamente às mudanças das condições ambientais. Estudos anteriores sugeriram que a via “High Osmolarity Glycerol” (HOG) MAPK e a produção de ceramida estão envolvidas na morte celular apoptótica de leveduras induzida por ácido acético. Há também evidências que alterações nos níveis de ceramidas endógenas podem afectar o destino de células de levedura. No entanto, ainda não há um conhecimento exacto sobre a base molecular da morte celular induzida por ácido acético, sobre as mudanças induzidas ao nível celular pela ceramida, bem como as vias de sinalização envolvidas nestes processos. Neste trabalho, procurou-se elucidar o papel das vias de sinalização MAPK na resposta celular à ceramida e na morte celular induzida por ácido acético. Células tratadas com o análogo solúvel da ceramida N-acetil-D-esfingosina (C2-ceramida) mantiveram a integridade da membrana plasmática e não produziram espécies reactivas de oxigénio detectadas através da incubação com DHE. Numa cultura exposta a C2-ceramida, uma subpopulação das células morreu, enquanto outra parte da cultura cresceu, não se observando assim na placa uma redução de Cfus em relação ao tempo zero. Alguns mutantes deletados em componentes das vias MAPK mostraram subpopulações menos sensíveis do que a estirpe selvagem e assim consideraram-se como sendo estirpes mais resistentes à C2-ceramida, nomeadamente o mutante *wsc3*Δ. Os mutantes *wsc2*Δ, *wsc3*Δ, *ste20*Δ, *slt2*Δ e *mkk1*Δ/*mkk2*Δ foram significativamente mais resistentes ao ácido acético do que a estirpe selvagem. Esses mutantes mostraram também uma menor percentagem de células exibindo uma acumulação de ROS e uma maior percentual de células com integridade da membrana plasmática preservada do que a estirpe selvagem. Os genes *WSC2* e *WSC3* codificam sensores membranares na via MAPK da integridade da parede, sugerindo um papel para esta via de sinalização tanto na morte celular induzida por ácido acético como nas alterações induzidas por ceramida. Não é conhecida a ligação entre morte celular induzida por ácido acético, mas a peroxidação lipídica pode ser uma hipótese provável.

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Abbreviations

MAPK	Mitogenic Activated Protein Kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MAPKKKK	MAP kinase kinase kinase kinase
HOG	high osmolarity glycerol
CWI	Cell wall integrity
GPCR	G protein-coupled receptor
PAK	p21-activated kinase
MCA1	metacaspase 1
AIF	apoptosis-inducing factor
MOMP	mitochondrial outer membrane permeabilization
cyt *c*	cytochrome *c*
AAC	ADP/ATP carrier proteins
LCB	long-chain base
C2-ceramide	N-acetyl-D-sphyngosine (C2-ceramide
H_2O_2	hydrogen peroxide
ROS	reactive oxygen species
PI	propidium iodide
DHE	dihydroethidium
c.f.u.	colony forming units
$\Delta\Psi m$	mitochondrial membrane potential

Chapter 1

INTRODUCTION

This chapter contains sections from the publications:

C2-phytoceramide sensitizes S. cerevisiae cells to osmotic stress. Pacheco A, Azevedo F, Chaves SR, Côrte-Real M, Sousa MJ (*in preparation*)

Vacuole-mitochondrial cross-talk during apoptosis in yeast: a model for understanding lysosome-mitochondria-mediated apoptosis in mammals. Sousa MJ, Azevedo F, Pedras A, Marques C, Coutinho OP, Preto A, Gerós H, Chaves SR, Côrte-Real M. Biochem Soc Trans. 2011 Oct; 39(5):1533-7.

1. Introduction

1.1 Cell death

Cell death plays an important role in the maintenance of tissue homeostasis, and can be defined as an irreversible loss of plasma membrane integrity [1]. Two forms of cell death have been described more frequently: apoptosis and necrosis.

Necrosis is a type of cell death defined by morphological features, such as increasing the volume of the cytoplasm, plasma membrane rupture, swelling of cytoplasmic organelles (mitochondria, endoplasmic reticulum, etc..) and a moderate chromatin condensation [1]. It is traditionally defined as a form of cell death that is uncontrolled, chaotic, and accidental, due to exposure to extreme environmental conditions [2]. Recently, this assumption has been challenged and in some instances necrosis is now seen as a regulated, programmed process [2-4].

Apoptosis is a mechanism that deals with the homeostatic removal of cells that have been mutated, infected or are simply superfluous in multicellular organisms. Several diseases can be linked to poor regulation of apoptosis, including AIDS, cancer, and neurodegenerative disorders. Apoptosis is a mechanism of programmed cell death accompanied by morphological characteristics such as cell rounding, reduction in cellular volume, condensation and fragmentation of chromatin, and the formation of cell fragments (apoptotic bodies) [1]. The process of apoptosis ensures that cells are quickly removed without rupture of the plasma membrane, thus preventing inflammation of the environment. Since deregulation of apoptosis is associated with severe human pathologies, the identification of components of the different apoptotic pathways and the understanding of mechanisms underlying their regulation is critical for the development of new strategies for prevention and treatment against those diseases. Different biological models, including *Caenorhabditis elegans* and *Drosophila melanogaster*, were use as core models for cell death research. Yeast was not used in early apoptosis studies, since it was considered that there was no advantage for a unicellular organism to commit suicide. After the complete sequencing of the *Saccharomyces cerevisiae* genome, an exhaustive search for homologies in databases failed to uncover potential regulators of apoptosis, reinforcing the idea that this mechanism was not present in this yeast. However, although yeast is a unicellular organism, apoptosis could have an evolutionary advantage at the colony level. In the

wild, yeasts are multicellular colonies and not individuals, in which apoptosis may be a mechanism that saves and releases nutrients to the healthier cells. Thus, apoptosis acts as a mechanism of self-preservation of the colony as a whole [5]. Indeed an apoptotic pathway in yeast was later found in yeast as described in more detail in the next section. This knowledge, associated with the several attributes of yeast such as its rapid growth, its simple and inexpensive culturing, ease and safety in handling, and especially its well defined genetic system and easy genetic manipulation make this unicellular eukaryote an attractive model system for cell death research.

1.1.1 Yeast apoptosis

The first indication that there is a basic apoptotic mechanism in yeast came from the finding that a mutation in the *S. cerevisiae* gene *CDC48* results in cell death with characteristics of apoptosis such as DNA fragmentation, chromatin condensation, and exposure of phosphatidylserine on the outer plasma membrane [6]. Later, it was described that an apoptotic cell death can also be induced by H_2O_2 [7, 8], acetic acid [8, 9], hyperosmotic shock [8], exposure to UV radiation [10], viral "killer" toxins [11], pheromones [12], chronological [13, 14] and replicative aging [15], and in colonies on solid media [16]. In virtually all these stimuli, the type of cell death observed is dependent on the intensity of the applied stress, i.e., low or moderate doses lead to death by apoptosis, while high doses cause a cell death such as necrosis [7-11]. Afterwards, several genes involved in yeast apoptotic cell death were identified. The yeast protein Yor197w, with structural homology with mammalian caspases, was called yeast metacaspase 1 (MCA1) [17]. Key elements of apoptosis in mammalian cells, such as HtrA2/Omi, EndoG, and the apoptosis-inducing factor (AIF), are also present in the yeast genome [18]. The release of cytochrome *c* (cyt *c*) from mitochondria, another landmark of human apoptosis, also occurs in *S. cerevisiae* [19]. Moreover, several studies have determined that the expression of pro-apoptotic proteins in yeast can cause cell death, such as expression of human Bax [20, 21]. Co-expression of the anti-apoptotic proteins Bcl-2 or Bcl-xL in yeast cells can rescue the lethality caused by expression of Bax, indicating that at least some features of the regulation of mammalian apoptotic proteins are conserved in yeast [20].

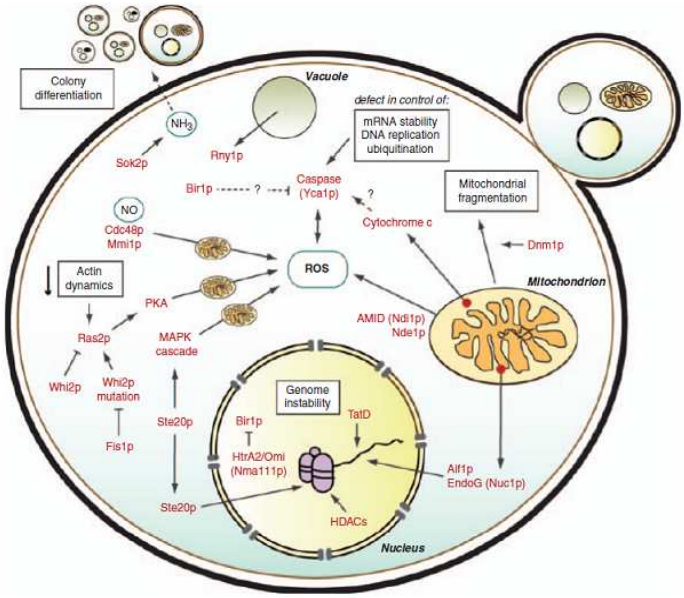

Fig. 1. Proteins and pathways in yeast apoptosis (adapted from [22])

In yeast, acetic acid-induced apoptosis is among the best-characterized apoptotic pathways, and, like in mammalian cells, mitochondria play a key role in this process. Indeed, different alterations in mitochondrial structure and function occurring during acetic acid-induced apoptosis have been identified. These changes include reduction in cristae number and mitochondrial swelling [23], a transient mitochondrial hyper- followed by depolarization, production of reactive oxygen species, decrease in cytochrome oxidase activity and mitochondrial outer membrane permeabilization (MOMP), with concomitant release of cyt *c* and yeast Aifp [19, 24]. Yeast orthologs of some of the mammalian permeability transition pore components were found to be involved in MOMP and cyt *c* release. While deletion of POR1 (yeast voltage dependent anion channel) enhances apoptosis triggered by acetic acid, CPR3 (mitochondrial cyclophilin) deletion has no effect. In contrast, absence of ADP/ATP carrier proteins (AAC), yeast orthologs of the adenine nucleotide transporter, protects cells exposed to acetic acid and impairs MOMP and cyt *c* release [25]. Mitochondrial proteins involved in fission/fusion, namely, Fis1p, Dnm1p, Mdv1p [26] have also been implicated in the execution of the yeast apoptotic program induced by acetic acid. Despite the large number of proteins shown to be involved in acetic acid-induced cell death, the upstream events and pathways that signal acetic acid-induced apoptosis, as well apoptosis induced by other stimuli, remain to be identified.

1.2 Ceramide signalling in *Saccharomyces cerevisiae*

Sphingolipids are the main lipid component in eukaryotes plasma membranes. The components of all sphingolipids are a long-chain base (LCB), a fatty acid, and a polar head group. The shingolipid ceramide acts as an important second messanger in mammalian cells. Indeed, rapid and transient changes in sphingolipid metabolism are associated with a wide range of cellular processes such as cell growth, differentiation, apoptosis, stress responses, and senescence. Ceramide and sphingosine levels increase in response to stress and in apoptosis induced by several stimuli such as FAS activation [27] and anticancer drugs [28]. Ceramides regulate mammalian apoptosis by transcriptional-dependent and -independent mechanisms. The transcriptional-independent mechanism of ceramide-mediated apoptosis involves generation of reactive oxygen species and release of mitochondrial intermembrane space proteins, with a pro-apoptotic role and is inhibited by anti-apoptotic members of the Bcl-2 family [29]. The former involves transcriptional activation of different signalling cascades including the c-Jun-N-terminal Kinase (JNK) pathway, also called the Stress Activated Protein Kinase (SAPK) pathway, which requires Ras and Rac1 [30]. Other intracellular targets of ceramide include kinase suppressor of Ras, Raf1, PKC and the ceramide-activated protein phosphatases PP1 and PP2A. Ceramide can also promote the clustering of death receptors and interferes with the relay of PI3K signals by activating protein phosphatases, such as ceramide-activated protein phosphatase (CAPP) [31].

As mentioned above, the yeast *S. cerevisiae* has been extensively used in the elucidation of numerous cellular and molecular processes shared across species, such as cell cycle control and apoptosis [32]. Several studies indicate that the ceramide pathway is a ubiquitous and conserved signalling system from yeast to human [29]. Similarly to mammalian cells, yeast ceramide levels increase in response to diverse stress treatments [33] and different studies showed that perturbations in sphingolipid metabolism determine cell fate. Indeed, expression of mammalian sphingomyelin synthase (SMS1) suppresses Bax-mediated yeast cell death and confers increased resistance to different apoptosis inducers [34]. These results suggest that SMS1, which uses ceramide to synthesize sphingomyelin, protects cells against death by counteracting stress-induced accumulation of the pro-apoptotic ceramide. Very recently, it has also been reported that exogenous N-acetyl-D-sphyngosine (C2-ceramide) can trigger a mitochondria-mediated

cell death process [35]. On the other hand, a different study showed that overexpression of *YDC1*, coding for a dihydroceramidase which hydrolyzes ceramide, results in reduced chronological lifespan and increased apoptotic cell death associated with fragmentation and dysfunction of mitochondria and vacuoles. These effects were reverted by exogenous addition of ceramide to YDC1-overexpressing cultures [36], suggesting ceramide can also have a protective effect in the cell. Yet another study showed that a yeast mutant deficient in Isc1p, a member of the family of the neutral sphingomyelinases, displays increased apoptotic cell death in response to hydrogen peroxide and during chronological aging [37]. More recently, a lipidomic approach revealed that these phenotypes were associated with increased levels of dihydro-C26-ceramide and phyto-C26-ceramide, again suggesting that ceramide signalling is indeed involved in yeast programmed cell death [38]. Exposure to C2-ceramide, a synthetic cell-permeable ceramide analog, specifically inhibits proliferation of *Saccharomyces cerevisiae* cultures in a dose-dependent manner and increases a serine/threonine phosphatase activity in cell crude extracts with biochemical characteristics similar to those of mammalian ceramide-activated protein phosphatase (CAPP) [39]. Yeast CAPP, composed of one catalytic subunit, Sit4p, and two regulatory subunits, Tpd3 and Cdc55, was subsequently found to mediate the proliferation arrest induced by C2-ceramide by blocking cells at the G1 phase of the cell cycle [40]. More recently, it was found that deletion of *SIT4* reverts the premature ageing and hydrogen peroxide sensitivity of *isc1*Δ cells and eliminates the respiratory defects and catalase A deficiency exhibited by this mutant [38]. Therefore, activation of Sit4p is involved in the mitochondrial dysfunction induced by increased levels of dihydro-C26-ceramide and phyto-C26-ceramide and leads to the shortened chronological lifespan and oxidative stress sensitivity of the *isc1*Δ mutant. These results provided the first glimpse of how changes in the levels of endogenous ceramides can affect cell fate in yeast. However, knowledge on the molecular basis of ceramide-induced cell changes, as well as of the role of signalling pathways in this process, is still lacking.

1.3 MAP kinase pathways in the yeast *Saccharomyces cerevisiae*

Mitogenic Activated Protein Kinase (MAPK) cascades are important signalling pathways (ubiquitous in eukaryotes) that allow yeast cells to swiftly adapt to changing environment conditions. These pathways regulate various important processes, from cell proliferation and differentiation to cell death. MAPK cascades normally contain three protein kinases that act in sequence: a MAP kinase kinase kinase (MAPKKK, MAP3K, MEKK or MKKK), a MAP kinase kinase (MAPKK, MAP2K, MEK or MKK), and a MAP kinase (MAPK). Therefore, when the cascade is activated, the MAPKKK phosphorylates the MAPKK which in turn phosphorylates both the threonine and tyrosine residues of a conserved -Thr-X-Tyr- motif within the activation loop of the MAPK [41].

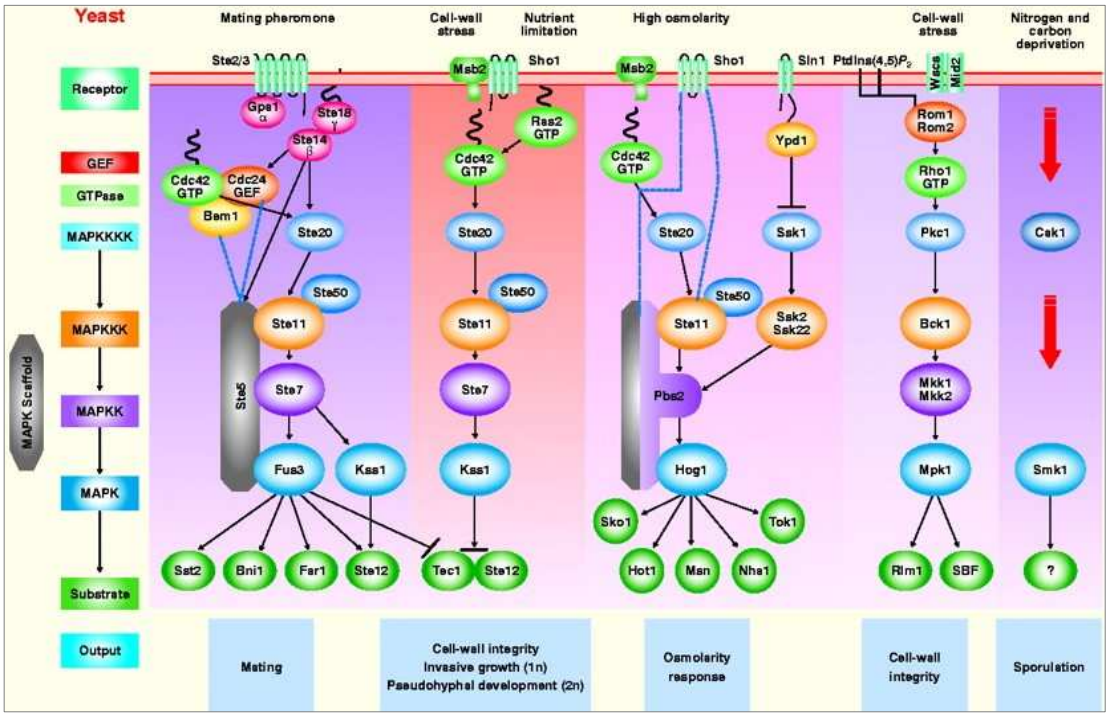

Fig. 2. Overview of the MAPKinase pathways in yeast (adapted from [42])

MAPKs phosphorylate a diverse set of well-characterized substrates, including transcription factors, translational regulators, MAPK-activated protein kinases (MAPKAP kinases), phosphatases, and other classes of proteins, thereby regulating metabolism, cellular morphology, cell cycle progression, and gene expression in

response to a variety of extracellular stresses and molecular signals [43]. The specificity of the MAPK pathways is regulated at several levels, including kinase-kinase and kinase-substrate interactions, colocalization of kinases by scaffold proteins, and inhibition of cross-talk/output by the MAPKs themselves [44]. *S. cerevisiae* contains five MAPKs, Fus3p, Kss1p, Hog1p, Slt2/Mpk1p and Smk1p, on five functionally distinct cascades, associated with the pheromone-mating response, invasive growth/pseudohyphal development, high osmolarity, cell wall integrity, and sporulation, respectively [45]. The five MAP kinases are controlled by four MAPKKs, Ste7p (regulating Fus3p and Kss1p), Pbs2p and the redundant pair Mkk1p/Mkk2p, and by four MAPKKKs, Ste11p, the redundant pair Skk2p/Skk22p and Bck1p. The specificity of signal transduction is guaranteed by scaffold proteins [46], Ste5p for the pheromone-mating response pathway, and Pbs2p for the HOG pathway.

1.3.1 The pheromone/mating response pathway

S. cerevisiae can exist as either diploid or haploid cells. Haploids can be of two mating types, *MATa* or *MATα*. Cells of opposing mating types can mate, by undergoing cellular and nuclear fusion, originating a diploid cell *MATa*/*MATα*. This process is initiated by the release of mating pheromones **a**-factor and **α**-factor, from respectively *MATa* and *MATα* cells, which act on cells of the opposing mating type. The **a**-factor interacts with *MATα* cells by binding to the G protein-coupled receptor (GPCR) Ste3p; in turn the **α**-factor interacts with *MATa* cells by binding to GPCR Ste2/3p [47, 48]. Binding of the mating pheromones activates the plasma membrane GPCRs, Ste2p and Ste3p, which in turn induces the dissociation of the heterotrimeric G protein Gpa1p-Ste14p-Ste18p, in Gα (Gpa1p) and the Gβγ complex (Ste14p-Ste18p) [49]. The Gβγ complex then activates the downstream proteins Ste20p and Ste5p. It is important to point out that Ste20p, a member of the p21-activated kinase (PAK) family of protein kinases, activates three of the yeast MAPK pathways, namely pheromone-mating response, invasive growth/pseudohyphal development, and high osmolarity. In these pathways, Ste20p is activated by the Small rho-like GTPase Cdc42p, and activated Ste20p is responsible for the phosphorylation and activation of Ste11p. Cdc4p2 localizes to the plasma membrane, and Ste20p is brought to its vicinity via its binding to Bem1p, an adaptor protein [50]. Likewise, Set11p is recruited to the same vicinity as activated

Ste20p because it interacts with the adaptor protein Ste50p, which in turn can associate with Cdc42p, thus connecting Ste11p to Ste20p at the plasma membrane [51, 52]. Activated Ste11p phosphorylates and activates the MAPKK Ste7p, which in turn phosphorylates and activates the MAPK Fus3p. The signal transduction along the pathway is mediated by the scaffold protein Ste5p, which binds both the Gβγ complex and all three component kinases of the pheromone-mating response MAPK cascade Ste11p, Ste7p, Fus3p [53, 54].

The cellular responses to pheromones and the subsequent actions elicited by the MAPK Fus3p include cell cycle arrest in G1, polarized cell growth to form cellular projections ("shmoos") towards a mating partner, expression of proteins required for cell adhesion, cell fusion (plasmogamy), and nuclear fusion (karyogamy).

1.3.2 Invasive growth/pseudohyphal development pathway

In environments were nutrients become limiting, yeast cells will undergo morphological changes, becoming more elongated, and proliferate in a unipolar pattern. Cells growing under those conditions exhibit increased cell-cell adhesion, cell-substratum adhesion, and increased capacity to penetrate the substratum [43]. These sets of changes induced by nutrient limitation are named invasive growth in haploids (elicited by glucose limitation) and pseudohyphal development in diploids (elicited by nitrogen limitation) [55]. The switch from a normal yeast state to an invasive growth/pseudohyphal development involves a signalling pathway very similar to the pheromone-mating response pathway [56]. This pathway is comprised of a MAPK cascade that mediates signal transduction from two GTP binding proteins; active Ras2p (yeast homologous of mammalian H-Ras), which mediates activation of Cdc42p [56, 57]. In this pathway, several transmembrane proteins are necessary to elicit filamentous growth: Sho1p, Msb2p, Mep2p and Gprp [58]. Sho1p can form a complex with Msb2p, and absence of either protein blocks activation of the MAPK Kss1p and prevents filamentous growth [58]. As in the pheromone-mating response pathway, Cdc42p is required for the function of the PAK Ste20p. The subsequent MAPkinase module is basically the same, except the scaffold protein Ste5p from the pheromone-mating response pathway is absent.

1.3.3. Cell wall integrity pathway

The yeast cell wall is a strong and rigid barrier that protects cells from extreme changes in the environment. The yeast cell wall has four major functions: stabilization of internal osmotic conditions, protection against physical stress, maintenance of cell shape, which is a precondition for morphogenesis, and its function as a scaffold for proteins [59]. It consists of an inner layer of load-bearing polysaccharides (glucan polymers and chitin), acting as a scaffold for a protective outer layer of mannoproteins that extend into the medium [59]. The inner layer is composed mainly (80 to 90%) of β1,3-glucan chains with some β1,6-linked glucan branches. Polymers of β1,6-glucan chains make up most of the remainder of the inner layer (8 to 18%), with chitin chains representing the smallest fraction (1 to 2%) [59]. The Cell Wall Integrity (CWI) signalling pathway is the predominant signalling pathway employed by yeast *S. cerevisiae* to maintain cell wall integrity during growth and development and to cope with cell wall stresses caused by external stimulus.

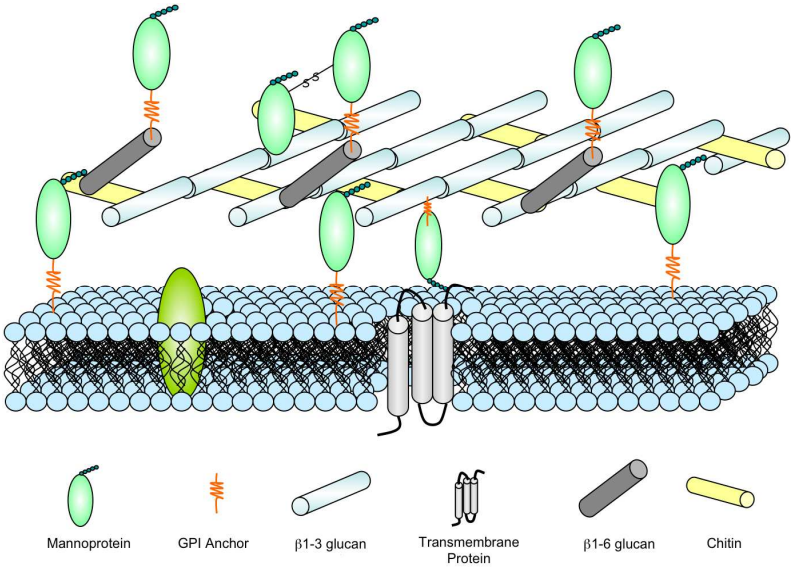

Fig. 3. Yeast cell wall (adapted from [103])

The CWI pathway is regulated by the cell cycle and is activated by several cell wall stresses caused by external stimuli (heat stress, hypotonic stress, mating pheromone, cell wall stressing agents, oxidative stress, actin cytoskeleton depolarization, etc). This MAP kinase cascade consists of five cell surface sensors, Wsc1p (aka Slg1, Hcs77),

Mid2p, Mtl1p, Wsc2p and Wsc3p, coupled to the GTP-binding protein Rho1p [60], which activates an array of effectors: Pkc1p, an essential activator (MPKKKK) homologous to the mammalian PKC isoforms, the MAPKKK Bck1p, the redundant pair of MAPKK Mkk1/2p and the MAPK Slt2p also known as Mpk1p [61].

Pkc1p was the first component of this pathway identified. *pkc1*Δ mutants show a growth defect that can be rescued by an osmoregulator (1 M sorbitol) at 30ºC or lower temperatures [62]. Mutants in components of the MAP kinase downstream of Pkc1p showed a similar phenotype to *pkc1*Δ mutants, though less severe. For instance, the growth defect of a *bck1*Δ mutant strain was rescued by 1 M sorbitol even a 37ºC, suggesting that Pkc1p regulates others targets beside the MAP kinase cascade [63]. Roh1p, a G-protein homologous to mammalian RhoA [64], is considered the prime regulator of CWI signalling. In *S. cerevisiae*, there are four additional related GTP-binding proteins: Rho2p (53% identity to Rho1), Cdc42p (50%), Rho3p (44%), and Rho4p (38%) [65]. Rho proteins alternate between the active GTP-bound state and the inactive GDP-bound state. They are regulated both by GTPase-activating proteins (GAPs) and guanosine nucleotide exchange factors (GEFs), acting in opposition. Rho1p is regulated by four GAPs - Bem2p, Sac7p, Bag7p and Lrg1p - and is stimulated through the action of the Rom1p and Rom2p GEFs [65, 66]. Four effectors of Rho1p have been described: the Pkc1p protein kinase, the 1,3-β-glucan synthase (GS), the Bni1p and Bnr1p formin proteins, and the Skn7p transcription factor (activated in the Sln1p branch of the HOG pathway) [67].

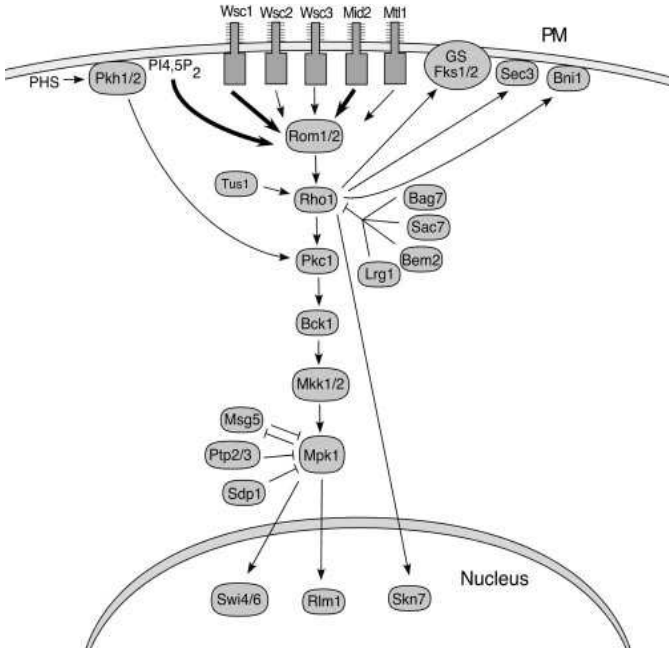

Fig. 4. Cell wall integrity (CWI) signalling pathway (adapted from [61]).

The MAPKinase cascade of the CWI pathway mediates transcriptional responses by way of two regulators, the SBF (Swi4/Swi6p) complex and Rlm1p, which are targets of phosphorylation by the MAPK Slt2/Mpk1p [61]. The SBF complex is composed of the DNA-binding subunit Swi4p and the regulatory subunit Swi6p [68]. This complex is vital in the G1 phase of the cell cycle and stimulates the periodic expression of the cyclin genes *CLN1*, *CLN2*, *PCL1*, and *PCL2*, as well as a large number of genes required for bud emergence, including genes encoding enzymes required for cell wall metabolism [68].

1.3.4 High Osmolarity Glycerol (HOG) pathway

In the natural habitat of yeast cells, a dramatic increase of the external osmolarity (hyper-osmotic shock) is a typical scenario. A common solution shared by different types of cells is accumulating solutes to balance the internal cellular osmotic pressure with the external environment. In the case of yeast cells, they increase the synthesis of glycerol in a mechanism referred to as the High Osmolarity Glycerol response. The HOG pathway contains two transmembrane proteins, Sho1p and Sln1p (an osmosensor) and two different inputs can lead to activation of Hog1p.

Under hypo-osmotic conditions, Sln1p is constitutively active and catalyzes autophosphorylation. Subsequently, Sln1p phosphorylates an intermediate protein, the

histidine phosphotransfer protein Ypd1p, which then phosphorylates the response regulators Skn7p and Ssk1p. Phosphorylation of Ssk1p prevents the interaction of this regulator with the redundant pair of MAPKKKs Skk2p/Skk22p [69]. Phospho-Skn7p is the active form of this transcriptional factor and it was shown that in a hypo-osmotic activation scenario it regulates the expression of *OCH1*, a cell wall related gene [70]. Sln1p-Ypd1p-Ssk1p forms a phosphorelay system. Phosphorelay systems comprise a histidine kinase protein (e.g. Sln1p) that transfers a phosphate group to an intermediate protein (e.g. Ypd1p), which then transfers the phosphate to a response regulator protein (e.g. Ssk1p) [71].

Under hyper-osmotic conditions, Sln1p is inactivated, allowing for an increase of unphosphorylated Ssk1p. Dephospho-Ssk1p is able to bind to and activate the pair Skk2p/Skk22p [72]. These MAPKKKs phosphorylate a MAPKK, Pbs2p, which in turn dually phosphorylates and activates the MAPK, Hog1p [73].

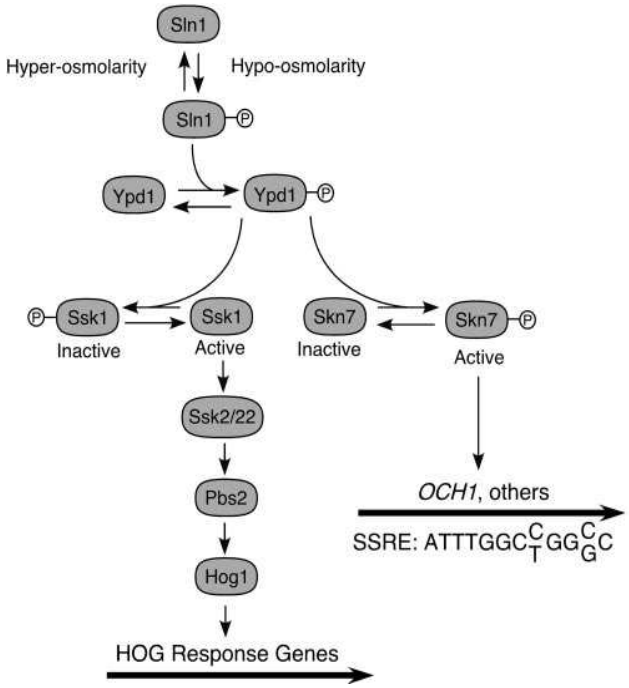

Fig. 5. The Sln1p branch of the HOG pathway (adapted from [61]).

The Sho1p branch is the second way by which Hog1p can be activated. Under hyper-osmotic conditions, Ste11p binds directly to Sho1p [74]. Ste11p then binds to the MAPKK Pbs2p, which in turn dually phosphorylates and activates Hog1p [74]. This causes its rapid accumulation in the nucleus, where it binds and phosphorylates several transcription factors. Hog1p also interacts with chromatin modifying enzymes and RNA

polymerase II, and affects the expression of hundreds of genes in response to hyperosmotic shock [75].

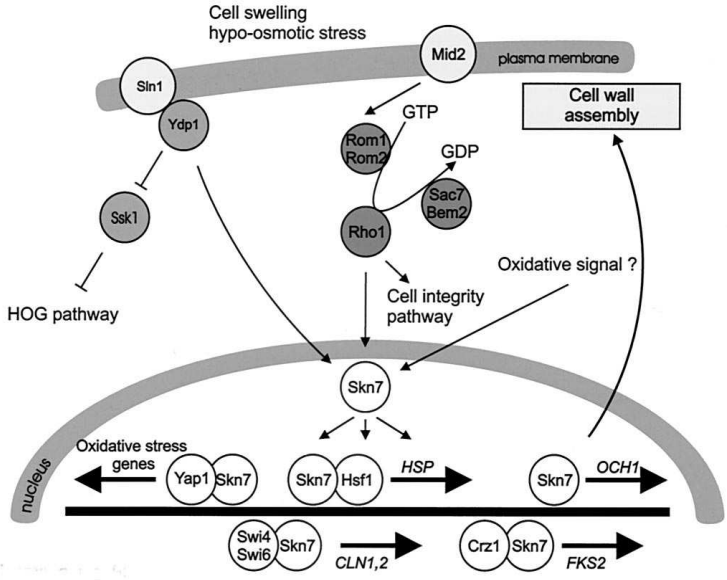

Fig. 6. Model for the role of Skn7p (adapted from [76]).

Is important to note that the transcription factor Skn7p has been implicated in the oxidative stress response, in cell cycle control, cell wall metabolism, and in the heat shock response, and seems to interact not only with the HOG pathway but also with the cell integrity pathway, the yeast Heat Shock Factor Hsf1, the Yap1p oxidative stress transcription factor, cell cycle-dependent transcription and calcineurin-dependent signalling [76].

Chapter 2

OBJECTIVES AND RESEARCH PLAN

2. Objectives and research plan

Expression of mammalian sphingomyelin synthase (SMS1) suppresses Bax-mediated cell death in yeast and confers increased resistance to different apoptosis inducers [34]. These results suggest that SMS1, using ceramide to synthesize sphingomyelin, protects against death by counteracting stress-induced accumulation of the proapoptotic ceramide. Data from our lab (unpublished) show that mutants in Isc1p, the yeast ortholog of mammalian neutral sphingomyelinase (N-SMase) are more resistant to acetic acid-induced cell death. These data suggest acid-induced apoptosis involves a ceramide-mediated pathway. However, it is not know if ceramide induces cell death in yeast, or if the regulatory mechanisms might involve conserved pathways such as mitogen activated protein kinase (MAPK) signalling. The involvement of these signalling pathways in acetic acid-induced apoptosis is also not known. We thus set out to characterize the effect of ceramide on yeast cell death, as well as the potential involvement of MAPK signalling pathways in this process and in acetic acid-induced apoptosis.

A) *Elucidation of signalling pathways in ceramide-induced apoptosis*

Rationale:

Ceramide, which is generated intracellularly in response to different stress stimuli acts as a second messenger in the initiation phase of apoptosis and appears to regulate apoptosis through two different mechanisms. One involves transcriptional activation of different signalling cascades, including the JNK pathway, and the other occurs via alteration of mitochondrial function. Yet, the molecular mechanisms underlying ceramide pro-apoptotic activity, including its targets and the signalling systems with which it interacts, are for the most part incomplete.

Objective:

This work aimed to identify points of crosstalk between ceramide and MAPK signalling pathways. The identification of these targets provides means to further characterize the ceramide-induced death pathway in yeast and will give information on new putative

targets in mammalian cells. It could also provide an association, at the signalling level, between yeast and mammalian apoptosis. Moreover, this data could also offer a link between yeast stress signalling networks and mammalian apoptosis, which would facilitate further research on apoptosis.

Research plan:

1) Characterization of ceramide-induced death in the *Saccharomyces cerevisiae* wild type strain W303

2) Construction of MAPK knock-out mutants in the W303 strain background

3) Characterization of ceramide-induced death in the mutant strains constructed in (2)

B) *Elucidation of signalling pathways in acetic acid-induced apoptosis*

Rationale:

In yeast, acetic acid-induced apoptosis is one of the best characterized yeast apoptotic pathways [9, 77]. It has been shown that expression of Bcl-xL decreased acetic acid induced apoptosis, which correlated with phosphorylation of Bcl-xL [77]. Pre-treatment of yeast cells expressing Bcl-xL with JNK inhibitor II renders the cells resistant to acetic acid-induced apoptosis and eliminates the Bcl-xL phosphorylated form. These results suggest there is a yeast pathway homologue to the mammalian SAPK/JNK, responsible for acid-induced Bcl-xL phosphorylation. The mammalian protein kinases p38 and SAPK/JNK have been shown to be structurally and functionally homologous to Hog1, the MAPK from High Osmolarity Glycerol (HOG) pathway of yeast [78]. Therefore, it is expected that they share similar regulatory mechanisms [79]. Recent data also suggest that acetic acid activates the HOG pathway [80], and also leads to phosphorylation of Slt2p, a MAPKK from the PKC cascade. These results suggest an intricate relation between PKC and HOG signalling in acetic acid-induced apoptosis. Still, the involvement of other MAPK pathways cannot be discarded.

Objective:

This work aimed to characterize the involvement of MAPK signalling pathways in acetic acid-induced cell death. Our working hypothesis was that acetic acid-induced apoptosis involves ceramide production, and it was expected that at least the MAPK mutants identified in (A) would exhibit an identical phenotype regarding their response to acetic acid apoptosis-inducing concentrations. Since sensors for acetic acid had not been identified so far, possible candidate plasma membrane proteins were studied in order to try to identify other putative upstream effectors of acetic acid-induced cell death. We expected to ascertain whether acetic acid directly, and/or through changes in sphingolipids, regulates proliferation and apoptosis through opposite survival and death signalling pathways, or whether the stress-activated MAP kinases, necessary for growth in the presence of acetic acid in yeast, were also involved in cell death. In addition, we anticipated unraveling any possible connections between the two processes.

Research plan:

1) Screen the yeast strains disrupted in genes coding for components of the MAPK pathways that have been constructed in (A) for increased pro- or anti-apoptotic activity by assessing decreased or increased sensitivity to acetic acid-induced cell death.

2) Test the strains presenting altered phenotypes for apoptotic markers, to determine at what level the mutations are interfering with the cell death process.

Chapter 3

MATERIALS AND METHODS

3. Materials and methods

3.1 Strains

The yeast *S. cerevisiae* strain W303-1A (*MATa, ura3-52, trp1Δ 2, leu2-3,112, his3-11, ade2-1, can1-100*) was used throughout this work as the wild type strain. Several mutant strains deleted in key components of the MAPK pathways were constructed by replacing the respective genes in the W303-1A strain with a kanMX4 disruption cassette (Table I). Each disruption cassette was amplified by PCR, from genomic DNA purified from the respective Euroscarf deletion strain, using the flanking primers A and D and the primers Kanrev and Kanfwd that anneal in the kanMX4 cassette, as described in the Saccharomyces Genome Deletion Project database (Fig. 7A) [81, 82, 104]. Cassettes were transformed into W303-1A, and transformants selected on YEPD plates containing 200 µg/ml geneticin (Sigma). Correct integration of the cassette was confirmed by PCR using primers α and δ that anneal upstream and downstream of the insertion and the primers KanB and KanC, that anneal in the kanMX4 cassette, respectively (Fig. 7B).

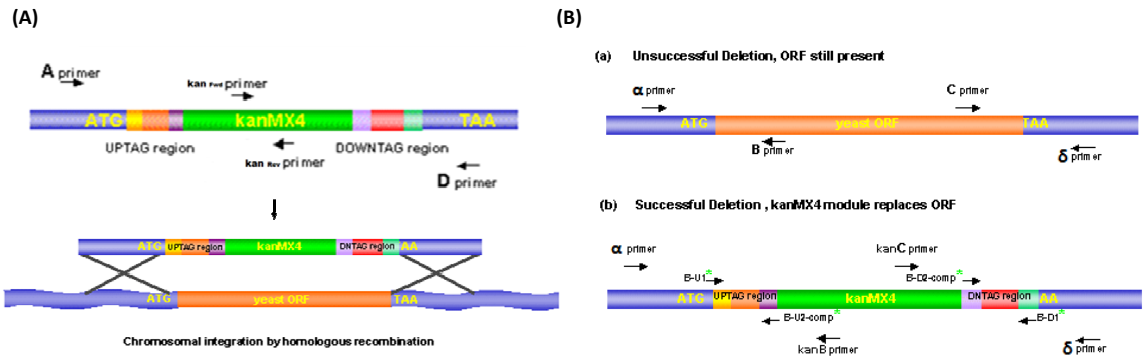

Fig. 7. Strategy used for the deletion of yeast ORFs (A) and confirmation of the correct integration of the deletion module (B) (adapted from [104]).

3.2 Growth conditions and treatments

Cells were maintained in rich medium (YPD) (1% yeast extract, 2% glucose, 2% bacto-peptone, 2% agar) and grown in synthetic complete medium (SC-Gal) (0.67% Bacto-yeast nitrogen base w/o amino acids (Difco), 2% galactose and 0.2% Dropout mix).

For ceramide treatment, yeast cells were grown as described above until exponential phase (OD_{600nm} – 0.5-0.6), harvested by centrifugation and suspended in fresh SC-Gal medium with 40 µM of N-acetylsphingosine (C2-ceramide, Sigma) dissolved in DMSO, or the equivalent volume of solvent (DMSO 0.1%, v/v) and incubated for 240 minutes at 30ºC with agitation (200 revolutions/min (rpm). Samples were taken at different time points, diluted to 10^{-4} in 1:10 serial dilutions in deionised sterilized water, and 40 µL drops were spotted on YPD agar plates in replicates of seven. Colony forming units (c.f.u.) were counted after 48 h incubation at 30°C. Cell viability was calculated as percentage of cfus normalized to the OD_{600} of the culture in relation to time zero. Cell viability for C2- ceramide treated cells was then normalized to the cell viability of DMSO-treated cells (see: Attachment II).

For acetic acid treatment, yeast cells were grown overnight in liquid SC-Gal until exponential growth-phase (OD_{600nm} – 0.5-0.6) at 30ºC with agitation (200 rpm). Cells were harvested by centrifugation and suspended in fresh SC-Gal medium (pH 3) with 120 mM acetic acid, and incubated for 200 minutes at 30ºC in 50 mL Erlenmeyer flasks with an air:liquid ratio of 5:1 in a mechanical shaker at 200 rpm. Samples were taken at different time points, diluted to 10^{-4} in 1:10 serial dilutions in deionised sterilized water, and 40 µL drops were spotted on YPD agar plates in replicates of seven. Colony forming units (c.f.u.) were counted after 48 h incubation at 30°C. Cell viability was calculated as percentage of cfus in relation to time zero.

3.3 Flow cytometry

During acetic acid and C2-ceramide treatments, samples were also taken to assess loss of plasma membrane integrity and production of reactive oxygen species (ROS) by flow cytometric analysis in an EPICS® XL™ (Beckman COULTER®) flow cytometer equipped with an argon-ion laser emitting a 488-nm beam at 15mW. Cells were collected by centrifugation, washed in deionised water, suspended in phosphate buffered saline (PBS) and stained with 1 µg/mL propidium iodide (PI, Sigma) or 2 µM/mL dihydroethidium (DHE, Sigma) for 30 min at room temperature, in the dark. Monoparametric detection of PI fluorescence was performed using FL-3 (488/620 nm) and detection of DHE was performed using FL-4 (488/675 nm).

Chapter 4

RESULTS

4. Results

Characterization of C2-ceramide induced cell death

Wild-type W303 cells were exposed to C2-ceramide for up to 240 min. As seen in figure 8, C2-ceramide did not lead to high levels of cell death. However, there was a decrease in cell viability of approximately 25% already after 60 min in comparison to the culture treated with DMSO, which did not increase much over time (Fig. 8B and 8C). Indeed, if results are not normalized to the OD_{600} to ensure that an equal number of cells were assessed, the percentage of cfu´s of cells exposed to C2-ceramide increases in relation to time zero, though not as much as for cells exposed to solvent alone (DMSO) (Fig. 8A). This indicates that the levels of cell death do not outweigh the growth of a sub-population of cells in the culture.

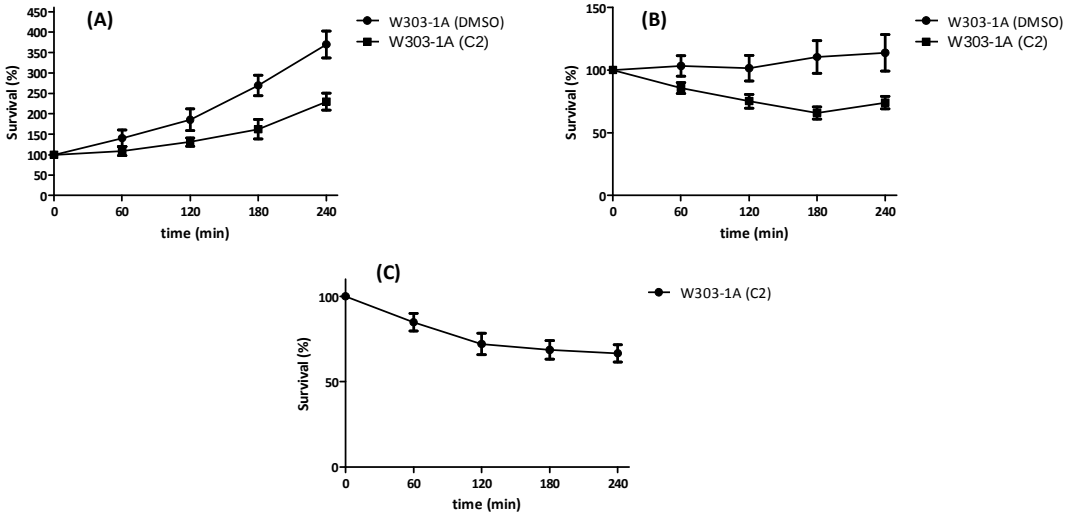

Fig. 8. The role of C2-ceramide in *S. cerevisiae* cell death. Wild-type yeast cells (W303-1A) were exposed to 40 µM C2-ceramide for 240 min. (A) Samples were taken at different time points, diluted and spotted on solid medium to asses viability. (B) The values of cfus were then normalized to the OD_{600} of the culture in relation to time zero. (C) Cell viability was calculate as the ratio between normalized % of viability of C2 and normalized % of viability of DMSO cultures (see: Materials and methods).

We thus sought to optimize treatment conditions in order to obtain higher levels of C2-ceramide induced cell death. We varied several parameters, such as temperature and time of incubation, medium, and adding additional C2-ceramide at different times. At higher temperatures, cells are often more sensitive to stress induced by different stimuli. However, C2-induced cell death was not enhanced when cells were incubated at 37ºC, rather than at 30ºC (Fig. 9A). Cell metabolism and composition of the medium can also affect the cellular response to external stimuli. Indeed, these parameters change during the growth of a culture; for instance, the pH lowers, and there are decreased levels of carbon source. Since cells were grown overnight to early-exponential phase, we sought to determine whether adding C2-ceramide to the growing cultures rather than resuspending cells in new media affected cell viability. However, this had no effect (not shown). We also hypothesized that C2-ceramide could be degraded after 60 min, as the loss in cell viability was not greatly enhanced from 60 to 240min. We therefore added additional ceramide at 60 min and 120 min after the initial treatment. As seen in figure 9B and 9C, this also had no effect on cell viability. We therefore could not optimize conditions that lead to high levels of C2 ceramide-induced cell death. Nonetheless, we next aimed to elucidate the role of MAPkinase signalling pathways in this process.

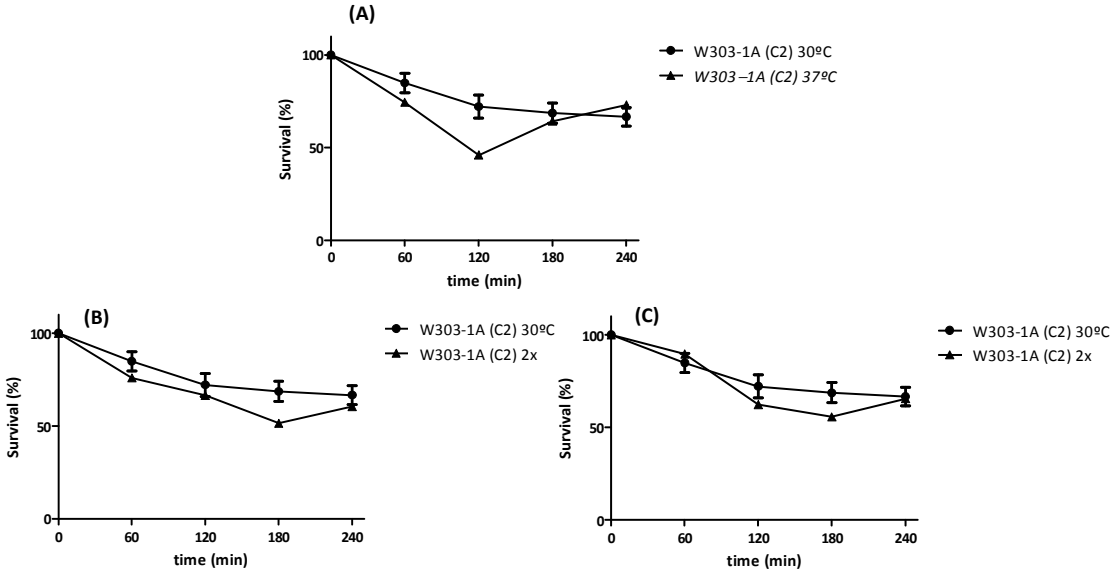

Fig. 9. Optimizing ceramide-induced cell death. Wild-type yeast cells (W303-1A) were exposed to different conditions: (A) Exposure to C2-ceramide at 30ºC and 37ºC; (B) addition of C2-ceramide at T0 and T60 min; (C) addition of C2-ceramide at T0, T60 and T120 min.

Construction of deletion mutants in components of the MAPK pathways

Several genes encoding components of the MAPK pathways were deleted in the strain background W303-1A. These genes are listed in Table 1 (Attachment I). Disruption cassettes were amplified by PCR from genomic DNA purified from the respective BY4741 deletion strains (Euroscarf), using two pair of primers: YFG A primer/ Kanrev primer, Kanfwd primer/ YFG D primer (Fig. 10A). The W303-1A strain was transformed with the amplified disruption cassettes, and transformants selected on medium containing geneticin. Correct integration of the cassette was assessed by colony PCR using two pairs of primers (upstream or downstream of insertion/ in kanMX4): YFG α primer/ KanB or KanB1 primer, KanC or KanC3 primer/ YFG δ primer (Fig. 10B).

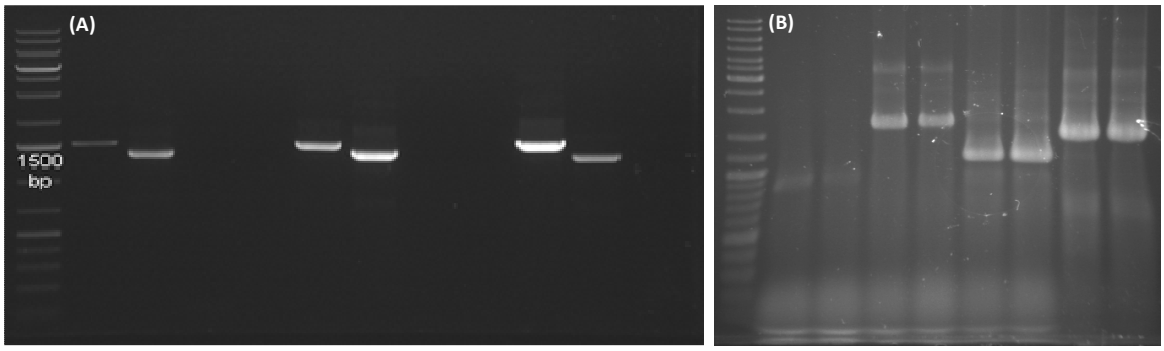

Fig. 10. Example of the PCR products obtained for the deletion of a yeast gene (*STE20*) (A) Disruption cassettes (B) confirmation of the correct integration of the cassette in W303 *ste20*Δ cells. BY4741 *ste20*Δ cells were used as a control. (Wells: **1-** W303 α ste20/KanB; **2-** BY4741 α ste20/KanB; **3-** W303 α ste20/KanRev; **4-** BY4741 α ste20/KanB; **5-** W303 KanC / δ ste20; **6-** BY4741 KanC / δ ste20; **7-** W303 KanFwd / δ ste20; **8-** BY4741 KanFwd / δ ste20).

Deletion mutants in components of the MAPK pathways are resistant to C2-ceramide induced cell death.

We proceeded to determine whether C2-induced cell death was altered in any of the MAPK mutant strains (*mid2*Δ, *sho1*Δ, *slg1*Δ, *sln1*Δ, *ssk1*Δ, *ste2*Δ, *ste3*Δ, *ste20*Δ, *wsc2*Δ, *wsc3*Δ, *hog1*Δ). In a preliminary experiment, *wsc3*Δ and *sho1*Δ (lacking a

membrane receptor of the cell wall integrity and high osmolarity-HOG pathways, respectively), and *hog1*Δ (lacking the MAPK of the high osmolarity-HOG pathway) mutants were slightly more resistant to C2 ceramide-induced cell death than wild type cells, indicating MAPK pathways may indeed be involved in ceramide signalling (Fig. 11). However further experiments will be necessary in order to confirm this hypothesis.

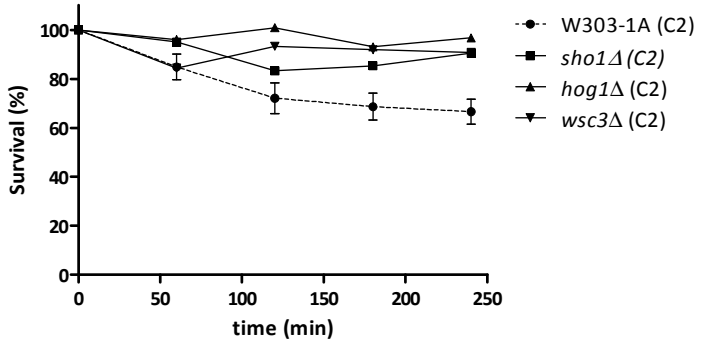

Fig. 11. The role of C2-ceramide in *S. cerevisiae* cell death. Survival of yeast cells (wild-type (W303-1A), *sho1Δ*, *hog1Δ* and *wsc3Δ*) exposed to 40 µM C2-ceramide for 240 min.

C2-ceramide does not lead to an increase in Reactive Oxygen Species or loss of plasma membrane integrity.

Most apoptotic stimuli lead to an increase in ROS levels in yeast [22], and mitochondria have proven key players in this process. On the other hand, a necrotic phenotype is characterized by a loss of membrane integrity. In order to characterize the nature of C2-ceramide induced cell death, we next determined whether exposure to C2-ceramide increased the levels of ROS and loss of plasma membrane integrity of both wild type cells and the mutant strains *sho1*Δ, *hog1*Δ and *wsc3*Δ. Cells were exposed to C2-ceramide as described above, and stained with DHE and PI, to measure accumulation of superoxide anion, and integrity of the plasma membrane, respectively. Fluorescence levels were measured by flow cytometry. For all strains tested, there were only residual levels of both ROS and PI staining, indicating C2-ceramide does not lead to an increase in ROS production, nor to increased loss of plasma membrane integrity. These results indicate C2-ceramide -induced cell death is not necrotic in nature, but do not point towards an apoptotic phenotype. Other apoptotic markers are under characterization.

Since C2-ceramide is not the equivalent of yeast endogenous ceramides, it is possible we cannot mimic the role of increased ceramide levels in yeast by adding exogenous C2-ceramide. As there is evidence that ceramide levels play an important role in acetic acid-induced cell death, we next aimed to further characterize the signalling pathways involved in the apoptotic phenotype triggered by acetic acid (another master thesis from our lab).

Components of the MAPK pathways modulate acetic acid-induced cell death.

In order to identify which genes are involved in acetic acid-induced cell death, we assessed the viability of the mutant strains described above after incubation with 120 mM of acetic acid for 200 min. Our results from a first screening revealed that acetic acid-induced cell death was not affected in the mutants *sho1*Δ, *wsc1*Δ aka *slg1*Δ, *ste2*Δ, *ste3*Δ and *ssk1*Δ, while *mid2*Δ cells displayed higher sensitivity (Fig. 12A). Also, our results show that the mutants *ste20Δ* (lacking a MAPKKKK, common to the mating, nutrient limitation and high osmolarity MAPK cascades), and *wsc2*Δ and *wsc3*Δ (lacking two membrane receptors of the cell wall stress-PKC pathway) were significantly more resistant to acetic acid-induced cell death than the wild type strain (Fig. 12B).

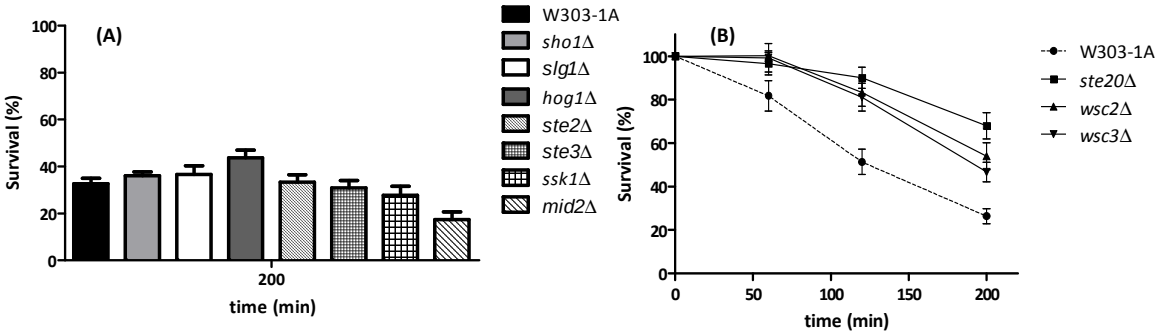

Fig. 12. The role of the MAPK signalling pathways in acetic acid-induced cell death. (A) Survival of the indicated yeast strains exposed to 120 mM acetic acid, pH3 for 200 min. Values represent means ± SD of one independent experiment with seven replicas. (B) Survival curves of the indicated strains exposed to 120 mM acetic acid, at pH3 for up to 200 min. Values represent means ± SD of at least three independent experiments.

These observations indicate that MAPK signalling pathways are involved in acetic acid-induced cell death, mainly through the Cell Wall Integrity (CWI) pathway. We therefore tested whether deleting other components of this pathway had an effect on acetic acid-induced cell death. Mkk1p/Mkk2p are a redundant pair of mitogen-activated kinase kinases in this signalling pathway which, upon activation by Bck1, phosphorylate its downstream target Slt2p. Wild type strain BY4741 and the mutant strains *mkk1*Δ*mkk2*Δ and *slt2*Δ were exposed to 120mM acetic acid, at pH3, for 200 min. As seen in figure 13, both strains were more resistant to acetic acid than their wild type counterpart, again indicating the CWI pathway is involved in signalling acetic acid-induced cell death.

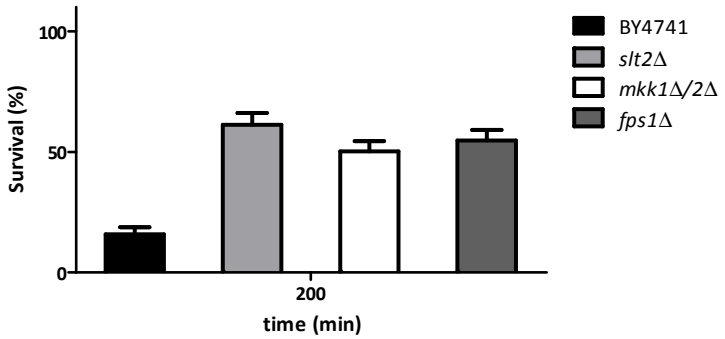

Fig. 13. The role of other components of the CWI MAPK signalling pathways in acetic acid-induced cell death. Survival of the wild-type (BY4741), *slt2Δ*, *mkk1*Δ*/mkk2*Δ and *fps1*Δ strains exposed to 120 mM acetic acid, at pH3 for 200 min, Values represent means ± SD of at least three independent experiments. The *fps1*Δ strain was used as a positive control.

Acetic acid induces a mitochondria-dependent apoptotic cell death in *S. cerevisiae* that displays characteristic apoptotic markers such as reactive oxygen species (ROS) overproduction, phosphatidylserine externalization, chromatin condensation, DNA fragmentation and mitochondrial dysfunction with release of cytochrome *c* [23].We next assessed whether the higher resistance of the *ste20*Δ, *wsc2*Δ and *wsc3*Δ strains to acetic acid was associated with a lower accumulation of ROS and higher preservation of plasma membrane integrity. These strains were incubated with 120 mM acetic acid, at pH 3 for 200 min, cells were stained with PI and DHE, and fluorescence analyzed by flow cytometry to assess loss of plasma membrane integrity and ROS accumulation, respectively. The *ste20*Δ, *wsc2*Δ, and *wsc3*Δ mutant strains had a lower percentage of cells displaying an accumulation of ROS and a higher percentage of cells with

preserved plasma membrane integrity than the wild type strain (Fig. 14). This is consistent with a role for Wsc2p and Wsc3p as sensor transducers in acetic acid-induced apoptosis signalling. Ste20p is involved in signal transduction of a number of pathways, so it remains to be determined whether its role in acetic acid-induced apoptosis is related to MAPK signalling and/or to other pathways.

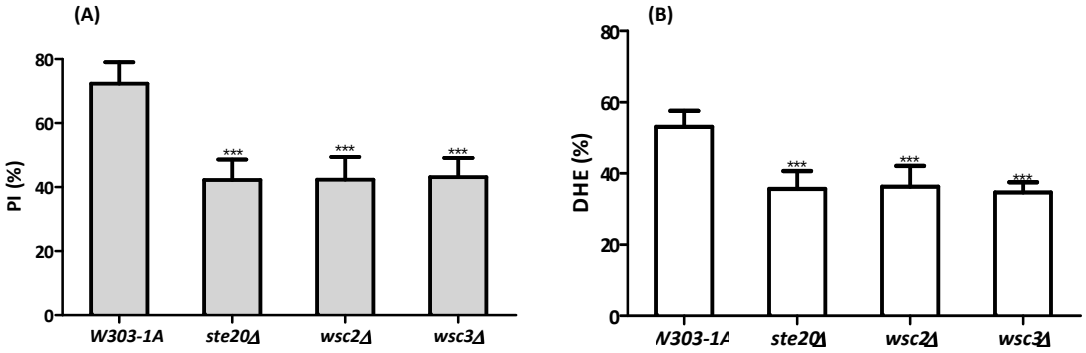

Fig. 14. Percentage of cells displaying PI internalization (A) and intracellular ROS levels (B) assessed by flow cytometry after treatment with 120 mM acetic acid, at pH3, for 200 min. Values are represented as means ± SD of at least three independent experiments with at least 20000 cells counted in each time point. Asterisks represent significance from control by one-way ANOVA test: $^{***}p < 0.001$.

The histone H2B S10 is involved in acetic acid-induced cell death

It has previously been reported that histone H2B is phosphorylated at S10 after exposure to apoptotic stimuli such as hydrogen peroxide, acetic acid, and α-factor, and inhibiting this phosphorylation event renders cells more resistant to these stimuli [83, 84]. The authors also showed that this phosphorylation is mediated by the Ste20p kinase *in vivo* and *in vitro* [83, 84]. This raised the hypothesis that the resistant phenotype observed in *ste20*Δ cells was simply due to a defect in phosphorylation of the histone H2B S10, and not to its role in the MAPK signalling pathways. This seems to be the case for H_2O_2-induced apoptosis, as the strains *ste20*Δ, H2B S10A (a strain with non-phosphorylatable H2B S10) and the double mutant H2B S10A *ste20*Δ were equally more resistant to H2O2-induced cell death than wild type cells. To test this hypothesis, we compared the viability of these strains (Wt, *ste20*Δ, H2B S10A, H2B S10A *ste20*Δ) after exposure to acetic acid. Unexpectedly, in our preliminary results the strain H2B

S10A had increased levels of plasma membrane integrity after exposure to acetic acid than strains *ste20*Δ or even H2B S10A *ste20*Δ (Fig. 15). This strain was constructed in this study, using the reagents provided by Dr Allis as described in Materials and Methods. We are currently deleting *STE20* in this strain background in order to exclude any alterations during the construction of this strain are responsible for the observed phenotype.

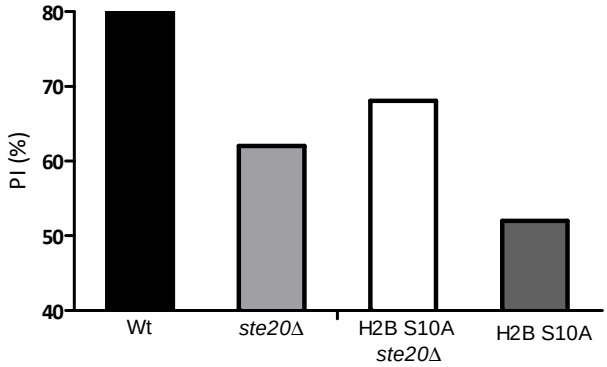

Fig. 15. Percentage of cells displaying PI internalization assessed by flow cytometry after treatment with 120 mM acetic acid, at pH3, for 200 min.

Chapter 5

DISCUSSION

5. Discussion

In this work, we investigated the possible involvement of the different MAPK pathways in *S. cerevisiae* ceramide and acetic acid-induced cell death. We intended to confirm whether acetic acid directly and/or through changes in sphingolipids regulates proliferation and apoptosis through opposite survival and death signalling pathways, and whether the stress-activated MAP kinases, involved in acetic acid resistance in yeast, are also involved in cell death. We also aimed to establish a link between the two processes. We started by determining whether exposure of the W303-1A strain to C2-ceramide resulted in cell death. We found that cells exposed to C2-ceramide showed a decrease in viability when compared with cells exposed to the solvent DMSO. However, cultures continued to grow in the presence of C2-ceramide. This seems to indicate that only a small fraction of the culture dies, whereas a large fraction of the cells survives and grows. Further optimization of the treatment conditions (temperature, ceramide concentration) did not increase C2-ceramide-induced cell death. Therefore, we next proceeded to determine if any of the mutant strains constructed showed altered phenotypes after incubation with C2-ceramide. Of all the mutants analyzed, *wsc3*Δ, *sho1*Δ and *hog1*Δ were slightly more resistant to C2 ceramide-induced cell death than wild type cells, indicating that signalling C2-ceramide-induced cell death may involve MAPK pathways. However, these results are very preliminary and warrant further confirmation. In the wild type strain or in these mutants, C2-ceramide did not induce loss of plasma membrane integrity, which would be indicative of necrosis, or an increase in intercellular ROS levels, which would suggest a mitochondria-mediated process. Therefore, the nature of C2-ceramide-induced death is still uncharacterized. The fact that C2-ceramide led to only a small reduction on cell viability further hampered our studies. It is known that sphingolipids are generated in response to heat stress in both mammals and yeasts [33, 85, 86]. It was also shown that C2-ceramide mimics the effects elicited by endogenously generated ceramide in the response of mammalian cells to different death stimuli [87]. However, it is possible that C2-ceramide is not the best molecule to use while studying the role of sphingolipid signalling in yeast apoptosis. Sphingolipids contain a long-chain base (LCBs in yeast are dihydrosphingosine (DHS) and phytosphingosine (PHS)), a fatty acid and a polar head group. LCBs are the best-characterized heat-induced sphingolipid signalling

molecules in yeast, whereas in mammals it is ceramide that signals cells to undergo apoptosis during severe heat stress and other stresses [33, 85, 86]. LCBs regulate various signalling pathways in S. *cerevisiae*, such as the Target Of Rampamycin Complex 2 (TORC2) and calcineurin, which in turn regulate ceramide synthase activity in response to heat and other stresses; *PKC1* is one of the downstream partners [86, 88]. It would be more appropriate to use the LCBs dihydrosphingosine and phytosphingosine to better define the cell death process mediated by sphingolipids. However, these molecules are not easily synthesized and produced in soluble form to allow their use in the proposed studies.

Regarding the characterization of the involvement of MAP kinase pathways in cell death induced by acetic acid, our results from a first screening revealed that acetic acid-induced cell death was not affected in the mutants *sho1*Δ, *wsc1*Δ aka *slg1*Δ, *ste2*Δ, *ste3*Δ and *ssk1*Δ, while *mid2*Δ cells displayed higher sensitivity. In contrast, *wsc2*Δ, *wsc3*Δ and *ste20*Δ strains, as well as *hog1*Δ cells, were significantly more resistant to acetic acid-induced cell death. These results are discussed bellow.

Mid2p is a plasma membrane sensor of the CWI pathway. The *mid2*Δ strain showed increased sensitivity to acetic acid-induced cell death. It has previously been shown that low extracellular pH conditions activate the CWI pathway via the cell sensor Mid2p [89]. In addition, Mid2p likely activates the transcription factor Skn7p [90], which has been associated with the control of oxidative stress response, among other roles [91-93]. Accordingly, deletion of *SKN7* sensitizes cells to oxidative stress [91-93]. Absence of Mid2p could result in lower activity of Skn7p, although Skn7p can function independently of Mid2p. It would therefore be interesting to determine whether *skn7*Δ cells are also more sensitive to acetic acid-induced cell death.

It has previously been shown that *hog1*Δ cells are more sensitive to growth on acetic acid-containing plates. In contrast, deletion of the aquaglyceroporin Fps1p, which mediates uptake of acetic acid, increases resistance to acetic acid of cells grown under the same conditions. The same authors showed that Hog1p phosphorylates Fps1p, targeting it for degradation. The phenotype of the *hog1*Δ*fps1*Δ mutant was indistinguishable from that of the *fps1*Δ strain, indicating Hog1p contributes to resistance of cells to chronic exposure to acetic acid through its role in lowering the levels of Fps1p, thus reducing the accumulation of intracellular acetic acid [94]. Unexpectedly, we found that the *hog1*Δ strain was more resistant to acetic acid than the

wild type strain. This may be explained by the different strain background used. However, it is also possible that deleting *HOG1* can provide enough energy to increase the capacity to repair damage in the short term. In oxidative stress, e.g. after exposure to acetic acid or H_2O_2, there is an increase in the levels of reactive oxygen species (ROS). ROS can cause oxidative damage in proteins, lipids and mainly in DNA, thus compromising cell viability. Cells have several defense mechanisms and invest energy to cope with the consequences of cellular damage, and express protective proteins or metabolites such as DNA damage repair enzymes, SOD, or catalase. Though a previous study showed that exposure to acetic acid did not alter catalase or superoxide dismutase activity in wild-type cells, acetic acid-induced cell death decreased in cells overexpressing catalase T and increased when Cu,Zn superoxide dismutase is overexpressed, suggesting that hydrogen peroxide contributes to acetic acid-induced cell death [95].

Osmotic stress leads to the production of glycerol, which is needed for osmotic adjustment. However, this requires a great deal of resources and energy. It has been shown that Hog1p plays a role in establishing the metabolic conditions for elevated glycerol production, as well as ensure there are adequate levels of enzymes necessary for glycerol production [96]. Therefore, deleting *HOG1* could prevent production of glycerol, thus increasing the available energy, which could then be used in recovering from damage. In the short term (up to 200 min, the length of our assay), reducing the resources channeled to produce glycerol could make resources available to the repair of damage. However, Hog1p would be required in the long run, thus rendering cells more sensitive to osmotic stress. Further studies will be required to test this hypothesis.

Ste20p is a MAPKKKK in the pheromone-mating response, invasive growth/ pseudohyphal development and HOG pathways, and is therefore not specific to one particular pathway. It is however interesting to note that it does not play a role in the CWI pathway. It is likely that the resistant phenotype observed in *ste20*Δ cells was simply due to a defect in phosphorylation of the histone H2B S10, and not to its role in the MAPK signalling pathways, which would indicate that acetic acid specifically induces the CWI pathway. This hypothesis is currently under investigation.

Wsc2p and Wsc3p function as plasma membranes receptors of the CWI pathway. Our initial results therefore suggested that this pathway is involved in signalling acetic acid-induced apoptosis, where Wsc2p and Wsc3p could function as sensors. We therefore tested other mutants lacking the MAPK and MAPKK of the CWI pathway, *slt2*Δ and

*mkk1*Δ/*mkk2*Δ, in which phosphorylation of downstream kinases is absent. These mutants were also more resistant to acetic acid-induced cell death. Our data therefore indicate that the CWI pathway is involved in signalling acetic acid- induced cell death, as blocking signal transduction in this pathway renders cells more resistant to acetic acid. The mutants *slt2*Δ, *mkk1*Δ/*mkk2*Δ, and *bck1*Δ are more sensitive to a variety of stimuli [76], as they have altered composition of the cell wall [76]. However, they are more resistant to oxidative stress. It would be interesting to further analyze which are the effectors of Stl2p, and the final targets of acetic acid-induced signalling. Slt2p (aka Mpk1p) targets the transcription factor complex SBF (Swi4p/Swi6p) and Rlm1p. The SBF complex is composed of the DNA-binding subunit Swi4p and the regulatory subunit Swi6p. In cell wall stresses, Fks2p (Catalytic subunit of 1,3-beta-glucan synthase) is induced, which requires the SBF complex. Indeed, activated (phosphorylated) Slt2p was detected in a complex with Swi4p and Swi6p at the *FKS2* promoter [97]. However, when cells are challenged with oxidative stress, Swi6p can also be directly oxidized in a specific cysteine residue (Cys404), resulting in a G1 delay while cells repair cellular damage. Swi6p can therefore function as an oxidative stress sensor for the regulation of the cell cycle, independently of Swi4p [98, 99]. It has been described that *swi6*Δ cells are more sensitive to chronic exposure to acetic acid, though its role has not been defined. It would be interesting to determine whether Swi4p, Swi6p, and Fks2p play a role in acetic acid-induced apoptosis, and whether it differs from that in chronic exposure to acetic acid. In a strain lacking Slt2p or lacking the ability to phosphorylate Slt2p (*mkk1*Δ/*mkk2*Δ), Swi6p could be more available to suffer direct oxidation, and thus could better control damage repair. This phenomenon, of enhanced damage repair by the greater availability of Swi6p maybe transient, because in the long term cell wall damage induced by acetic acid can be deleterious in cells lacking Slt2p (which by itself is very sensitive to various stresses), even though genes responsible for cell wall assembly can be controlled by other effectors. The need to fully block signal transduction in the CWI pathway to render a resistant phenotype to acetic acid should be tested further through studies in a *bck1*Δ strain.

The Hog1p pathway seems to be important for resistance to acetic acid at low pH. In accordance, a sub-lethal growth inhibitory concentration of acetic acid was shown to promote the phosphorylation of Hog1p and Slt2p [80]. However, from the 101 viable kinase mutants of the Euroscarf collection, only *hog1*Δ, *pbs2*Δ, *ssk1*Δ and *ctk2*Δ exhibited deficient growth in the presence of acetic acid. Activation of Hog1p by acetic

acid was shown to depend on the presence of *SSK1* and *PBS2*, but not of *SHO1* or *STE11*. In the same screening, loss of the cell integrity MAP kinase (Slt2p/Mpk1p) was found to slightly increase acetate resistance. In what concerns the known plasma membrane sensors of MAPK pathways, acetate-induced Hog1p activation appears to involve the Sln1p, whereas Slt2p activation was dependent on Wsc1p [100]. These data together with our results indicate that not all stress-activated MAP kinases involved in acetic acid resistance in yeast are also involved in acetic acid induced apoptosis.

The *wsc3*Δ mutant displayed a resistance phenotype to both ceramide and acetic-acid induced cell death. The higher survival of *wsc3*Δ, deficient in a sensor in the cell wall integrity MAPK pathway, suggests a role for this signalling pathway in both ceramide and acetic acid induced cell death. The link between acetic acid-induced cell death and cell wall integrity is not known, but lipid peroxidation by acetic acid may be a plausible candidate. Indeed, it has been shown that that linoleic acid hydroperoxide (LoaOOH), a product of radical attack on an unsaturated lipid, activates the CWI MAPK Slt2p (Mkk1p) [101].

Finally, in our study, exposure to acetic acid resulted in a high percentage of cells displaying loss of plasma membrane integrity, as well as high ROS production. This is in disagreement with an apoptotic phenotype but may be indicative of secondary necrosis following an initial apoptotic process. These markers should therefore be monitored at earlier time points under our experimental conditions. Other apoptotic markers should also be assessed in the future. However, there is growing evidence that necrosis is a controlled process. It has been described that necrotic programmed cell death in yeast is accompanied by typical morphological and cell functional changes. These include plasma membrane rupture, mitochondrial outer membrane permeabilization (MOMP), dissipation of mitochondrial potential, ATP depletion, overproduction of reactive oxygen species (ROS), and nuclear release of high mobility group box-1 (HMGB1) protein. In the future, it will be important to monitor necrotic markers such as the nuclear release of yeast HMGB1 (Nhp6Ap) [102]. Interestingly, Hsp90p has been functionally associated with regulation of necrosis and MAPKs such as Slt2p may be targets of Hsp90p, indicating MAPK pathways may also be involved in programmed necrosis [102].

Chapter 6

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Chapter 7

ATTACHMENTS

Attachment I : TABLE 1:

List of *S. cerevisiae* strains and recombinant plasmids used in this study

Strain	Genotype	Source/reference
W303-1A	*MATa*, *ura3-52*, *trp1Δ 2, leu2-3,112*, *his3-11*, *ade2-1*, *can1-100*	Euroscarf
W303-1B	*MATα*, *ura3-52*, *trp1Δ 2, leu2-3,112*, *his3-11*, *ade2-1*, *can1-100*	Euroscarf
YFlaAze01	W303-1A isogenic *gpd1 :: kanMX4*	This study
YFlaAze02	W303-1A isogenic *gpd2 :: kanMX4*	This study
YFlaAze03	W303-1A isogenic *rvs161 :: kanMX4*	This study
YFlaAze04	W303-1A isogenic *mid2 :: kanMX4*	This study
YFlaAze05	W303-1A isogenic *sho1 :: kanMX4*	This study
YFlaAze06	W303-1A isogenic *slg1:: kanMX4*	This study
YFlaAze07	W303-1A isogenic *sln1 :: kanMX4*	This study
YFlaAze08	W303-1A isogenic *ssk1 :: kanMX4*	This study
YFlaAze09	W303-1A isogenic *ste2 :: kanMX4*	This study
YFlaAze10	W303-1A isogenic *ste3 :: kanMX4*	This study
YFlaAze11	W303-1A isogenic *ste20 :: kanMX4*	This study
YFlaAze12	W303-1A isogenic *wsc2 :: kanMX4*	This study
YFlaAze13	W303-1A isogenic *wsc3 :: kanMX4*	This study
YFlaAze14	W303-1A isogenic *hog1 :: kanMX4*	This study
YFlaAze15	W303-1A isogenic *pkc1 :: kanMX4*	This study
JHY205	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT* pJH33[*CEN URA3 HTA1-HTB1 HHT2-HHF2*]	Ahn, et al [83]
JHY311	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT pQQ18[CEN LEU2 HTA1-HTB1 HHT2-HHF2]*	Ahn, et al [83]
SAY148	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ste20Δ::KAN hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT pQQ18[CEN LEU2 HTA1-HTB1 HHT2-HHF2]*	Ahn, et al [83]
SAY151	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ste20Δ::kanMX6 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT pSA17[CEN LEU2 HTA1-htb1-S10A HHT2-HHF2]*	Ahn, et al [83]
YFlaAze16	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT pSA17[CEN LEU2 HTA1-htb1-S10A HHT2-HHF2]*	This study
BY4741	*MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0*	Euroscarf
fps1Δ	BY4741 isogenic, *fps1 :: kanMX4*	Euroscarf
Slt2Δ	BY4741 isogenic, *slt2 :: kanMX4*	Euroscarf
Mkk1/2Δ	BY4741 isogenic, *mkk1 :: kanMX4*	Euroscarf

Attachment II

Equations

$$\%\ viability\ = \frac{cfus\ measured}{cfus\ \exp ected} \times 100 \qquad (1)$$

$$cfus\ \exp ected\ = \frac{cfus\ T_0 \times OD\ T_n}{OD\ T_0} \qquad (2)$$

$$\frac{ceramide}{DMSO} \times 100\% \qquad (3)$$